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## **GROWTH FACTORS AND TENSION-INDUCED SKELETAL MUSCLE GROWTH**

FINAL TECHNICAL REPORT and FINAL DISCLOSURE OF INVENTION REPORT

### **PRINCIPAL INVESTIGATOR**

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**FINAL DISCLOSURE OF INVENTION REPORT:** No inventions were conceived or reduced to practice during the course of work under this project

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**I. PROGRESS REPORT****A. Dates Covered By Project Since Last Competitively Reviewed:**

11/1/91 to present

**B. Previous Application's Specific Aims:**

Using tissue cultured skeletal avian myofibers in a computerized mechanical cell stimulator (MCS) device, we:

- a. Analyzed the mechanisms by which mechanical stimulation increased the myofiber's growth response to insulin-like growth factor-I (IGF-1);
- b. Analyzed the mechanisms by which mechanical stimulation reduced glucocorticoid-induced myofiber atrophy;
- c. Completed in-house modifications of the mechanical cell stimulator (MCS) model for space shuttle use, and performed centrifuge studies for takeoff/reentry simulation.

**C. Progress Toward Project's Goals:****(1). Insulin and IGF-1**

In order to analyze which growth factors are essential for stretch-induced muscle growth *in vitro*, we developed a defined, serum-free medium in which the differentiated, cultured avian muscle fibers could be maintained for extended periods of time. The defined medium (muscle maintenance medium, MM medium) maintains the nitrogen balance of the myofibers for 3 to 7 days, based on myofiber diameter measurements and myosin heavy chain content. Insulin and IGF-1, but not IGF-2, induced pronounced myofiber hypertrophy when added to this medium. In 5 to 7 days, muscle fiber diameters increase by 71% to 98% compared to untreated controls (73). Mechanical stimulation of the avian muscle fibers in MM medium increased the sensitivity of the cells to insulin and IGF-1, based on a leftward shift of the insulin dose/response curve for protein synthesis rates (54). Thus, one mechanism by which mechanical activity stimulates myofiber growth is by increasing the sensitivity of the cells to insulin-like growth factors. This mechanism is compatible with the known beneficial effects of exercise in patients with diabetes. The intracellular signalling mechanisms by which muscle stretch increases their sensitivity to insulin and IGF-1 was examined in FY 1993. Using an RIA, we measured IGF-1 efflux from the cultured avian muscle cells in response to stretch. Under static culture conditions the differentiated skeletal muscle cells were found to release large amounts of endogenous IGF-1 (1-3 nM) into the culture medium (54). When mechanically stimulated, the rate of IGF-1 release into the medium was significantly increased, but only during the first hours after initiating mechanical stimulation. Longer periods of stimulation led to a significant decrease in IGF-1 release from the cells. We tested several different mechanical activity patterns with similar results. This type of IGF-1 stretch response is compatible with a model whereby stretch increases the secretion but not the *de novo* synthesis of IGF-1 (54). Interestingly, addition of collagen (type I) to cultures of differentiated avian myofibers stimulated IGF-1 release from the skeletal muscle cultures 3 to 11 fold. Although IGF-1 has been shown by others to stimulate the synthesis of extracellular matrix molecules, this is the first time an extracellular matrix molecule has been found to stimulate the production of IGF-1. Since stretch is known to stimulate extracellular matrix production, the exogenous collagen which was used to embed the cells in our normal stretch protocol may mask any long term additional stretch effect on IGF-1 production.

The avian skeletal muscle cells also synthesize and secrete IGF-1 binding proteins which can influence the growth-stimulating properties of IGF-1. We developed a ligand binding assay for IGF-1 binding proteins and found that the avian skeletal muscle cultures produced three major

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species of 31, 36, and 43 kD molecular weight (54). Stretch of the myofibers was found to have no significant effect on the efflux of IGF-1 binding proteins, but addition of exogenous collagen stimulated IGF-1 binding protein production 1.5 to 5 fold (54).

## (2). Steroids

Steroid hormones have a profound effect on muscle protein turnover rates *in vivo*, with the stress-related glucocorticoids inducing rapid skeletal muscle atrophy while androgenic steroids induce skeletal muscle growth. Exercise in humans and animals reduces the catabolic effects of glucocorticoids and may enhance the anabolic effects of androgenic steroids on skeletal muscle. In our continuing work on the involvement of exogenous growth factors in stretch-induced avian skeletal muscle growth, we have performed experiments to determine whether mechanical stimulation of cultured avian muscle cells alters their response to anabolic steroids or glucocorticoids. In static cultures, testosterone had no effect on muscle cell growth, but 5 $\alpha$ -dihydrotestosterone and the synthetic steroid stanozolol increased cell growth by up to 18% and 30%, respectively, after a three day exposure. Mechanical stimulation did not alter the muscle cell's growth response to testosterone (12), and we are currently examining the interactions of dihydrotestosterone and stanozolol with mechanical stimulation.

The glucocorticoid dexamethasone induces atrophy of the differentiated cultured avian myofibers after 3 to 5 days of incubation (10). Mechanical stimulation of the muscle cells for 3 to 4 days in the presence of dexamethasone significantly attenuated this atrophic response, based on a 79% reduction in the dexamethasone-induced fall in protein/DNA ratios and myosin content. Thus, mechanical stimulation modifies the response of the muscle cells not only to insulin and IGF-1 but also to glucocorticoids. We have extended these observations to determine whether stretch attenuates dexamethasone-induced muscle atrophy by a prostaglandin-dependent mechanism. Dexamethasone inhibited the production of the anabolic prostaglandin F<sub>2 $\alpha$</sub>  in static muscle cultures and mechanical stimulation reversed this decrease (11). The stretch-induced increase in PGF<sub>2 $\alpha$</sub>  resulted partially from stretch activation of the enzyme responsible for synthesizing prostaglandins, cyclooxygenase. Thus, mechanically-induced protection of muscle fibers from the catabolic effects of stress-related hormones such as the glucocorticoids acts at the level of cyclooxygenase and could be an important mechanism by which exercise protects skeletal muscle from stress-related atrophy in space. Understanding the molecular mechanism by which stretch regulates cyclooxygenase activity and prostaglandin production could eventually lead to the development of beneficial pharmacological agents in the treatment of muscle wasting and is a major focus of FY 1994 studies.

## (3). Computerization of the mechanical cell stimulator and growth of myofibers in a flight certified bioreactor system

We completed development of a new IBM-based mechanical cell stimulator system to provide greater flexibility in operating and monitoring our experiments. Our previous long term studies on myofiber growth were designed around a perfusion system of our own design. We have recently changed to performing these studies using a modified CELLCO cartridge bioreactor system since it has been certified as the ground-based model for the Shuttle's Space Tissue Loss (STL) Cell Culture Module. The current goals of this aspect of the project are three fold: 1) to design a cell culture system for studying avian skeletal myofiber atrophy on the Shuttle and Space Station; 2) to expand the use of bioreactors to cells which do not grow in either suspension or attached to the hollow fibers; and 3) to combine the bioreactor system with our computerized mechanical cell stimulator to have a better *in vitro* model to study tension/gravity/stretch regulation of skeletal muscle size. The hollow fiber growth cartridges in the CELLCO system have been modified in our lab to accept differentiated skeletal muscle organoids, as described in (4) Preliminary Studies. In addition, long term growth of three dimensional skeletal muscle organoids on permeable biopolymers in bioreactor cartridges may be useful for subsequent *in vivo* transplantation for gene therapy studies, which is part of this renewal application. Combining the computerized mechanical

cell stimulators with the rapid nutrient flow of bioreactor systems, will provide a better ground-based and space-based model system for studying muscle growth regulation.

#### D. Preliminary Studies

##### (1). Release of tension induces rapid atrophy of tissue cultured avian skeletal muscle cells :

All our previous studies have emphasized mechanically-induced hypertrophy of skeletal myofibers. In order to begin examining how mechanical stimuli and growth factors interact to prevent or reverse decreased tension/microgravity-related myofiber atrophy, we determined whether release of tension on differentiated avian skeletal myofibers in tissue culture indeed causes atrophy, as seen in adult skeletal muscle *in vivo*. Seven day avian skeletal myofiber cultures were released from their resting tension by approximately 20%. Total noncollagenous protein and myosin heavy chain content of the cultures rapidly decreased by 40%-50% in three days (Figure 1). This "tension-release" atrophy may be analogous to hindlimb suspension-induced atrophy *in vivo*, and tissue cultured mammalian myofibers will be utilized as part of this renewal proposal to study the role of growth factors and mechanical stimulation in attenuating or reversing "tension-release" muscle atrophy in tissue culture.

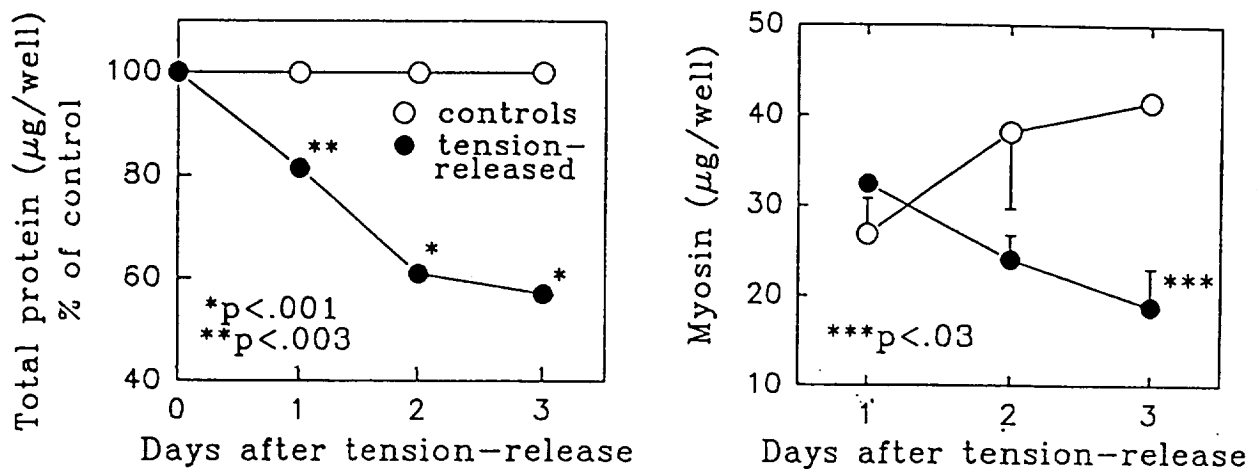
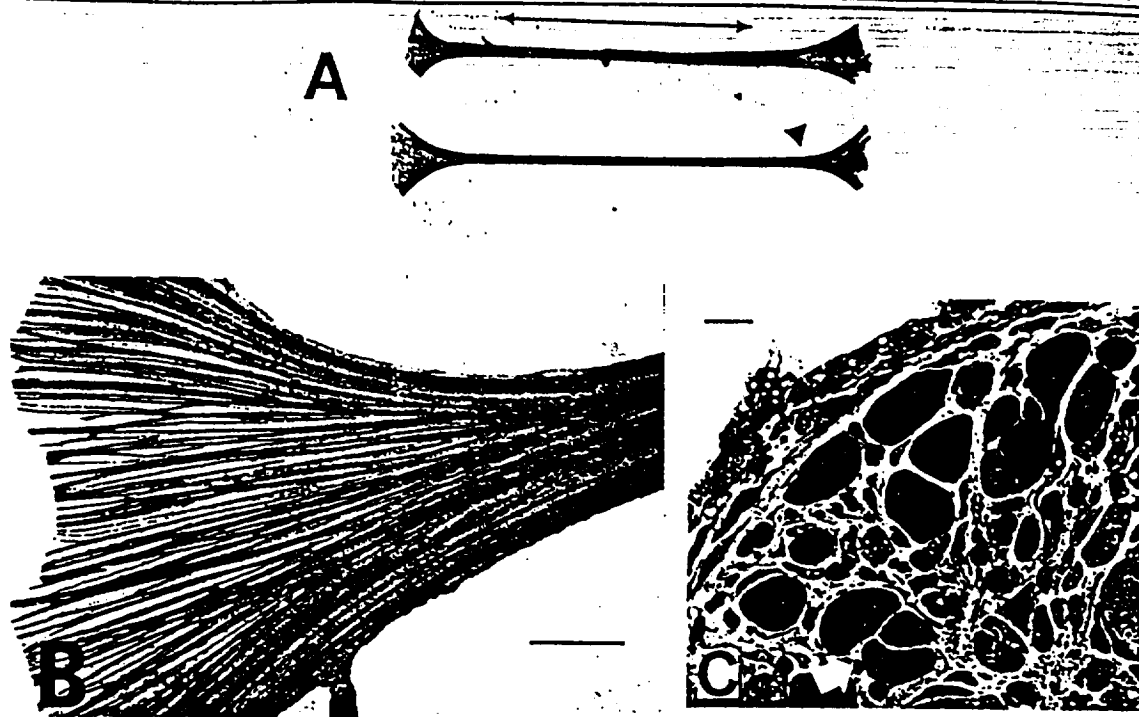


FIGURE 1. Tissue cultured skeletal myofibers (Day 7) released from their resting tension rapidly atrophy, as indicated by the rapid loss in total cellular protein (left) and myosin heavy chain (right).

##### (2). Avian skeletal muscle organoids can be transferred and maintained in modified cartridges of the Space Tissue Loss Module.

Avian skeletal muscle organoids (Figure 2), formed as outlined in APPENDIX PAPER 1 (79) in our Mechanical Cell Stimulator Model 2 (MCS-2, Figure 3), are difficult to maintain in positive nitrogen balance for extended periods of time (>14 days) under normal culture conditions in which the medium is changed manually every 24 h (79). Continuous medium perfusion would provide nutrients and growth factors in a more *in vivo*-like fashion, as well as prevent local build-up of harmful metabolic breakdown products. For continuous medium perfusion of muscle organoids, we have recently utilized the CELLMAX™QUAD Artificial Capillary Cell Culture System (CellCo, Gaithersburg, MD) for two reasons. First, this has been approved as the ground-based model for the Shuttle's Space Tissue Loss Cell Culture Module (STL). Second, we have been able to modify the cartridges used in the bioreactor to accept our skeletal muscle organoids. Avian muscle organoids developed in the elastic wells of our MCS-2 for 8-10 days, have a sterile stainless steel mesh bracket inserted into the wells above the organoid to maintain their length when removed from the



**FIGURE 2.** Computer-aided mechanogenesis of avian skeletal muscle organs from single cells *in vitro*. (A) Whole avian skeletal muscle organoids stained with either anti-fibronectin antibody (upper), or anti-tenascin antibody (lower). Double arrow shows direction of stretch; (B) Organoid end stained with anti-myosin heavy chain, showing parallel nature of myofibers in organoids; (C) Hematoxylin and eosin stained thin section of organoid showing development of muscle fascicles (white arrow), the functional unit of skeletal muscle. Photos are taken from reference (79).

MCS-2 support brackets (Figure 4). These organoids are well protected by the elastic substratum-stainless steel mesh, and have been centrifuged at 3 to 4 g's for 30 min in a laboratory centrifuge without evident morphological damage to the myofibers (unpublished observations). The elastic well, organoid, and bracket are transferred sterily to bioreactor cartridges. The cartridge modifications involve obtaining cartridge shells without hollow fibers from the manufacturer, and machining one end of the cartridge to accept a screw cap (Figure 5A). Up to six organoids can be placed into one cartridge (Figure 5B). Once the organoids are placed in the cartridge, they are mounted on the CELLMAX™QUAD bioreactor pump system and perfusion begun. Up to 4 cartridges (24 organoids) can be maintained at one time. Perfusion through the cartridges is at 1 to 5 ml/min, and daily measurements of perfusion medium glucose/lactate concentrations indicate the metabolic activity and growth status of the cells in the organoids (Figure 6). Protein turnover measurements are in progress to optimize long term maintenance of the avian muscle organoids. Similar studies are planned with the mammalian muscle organoids to be developed as part of this renewal proposal.

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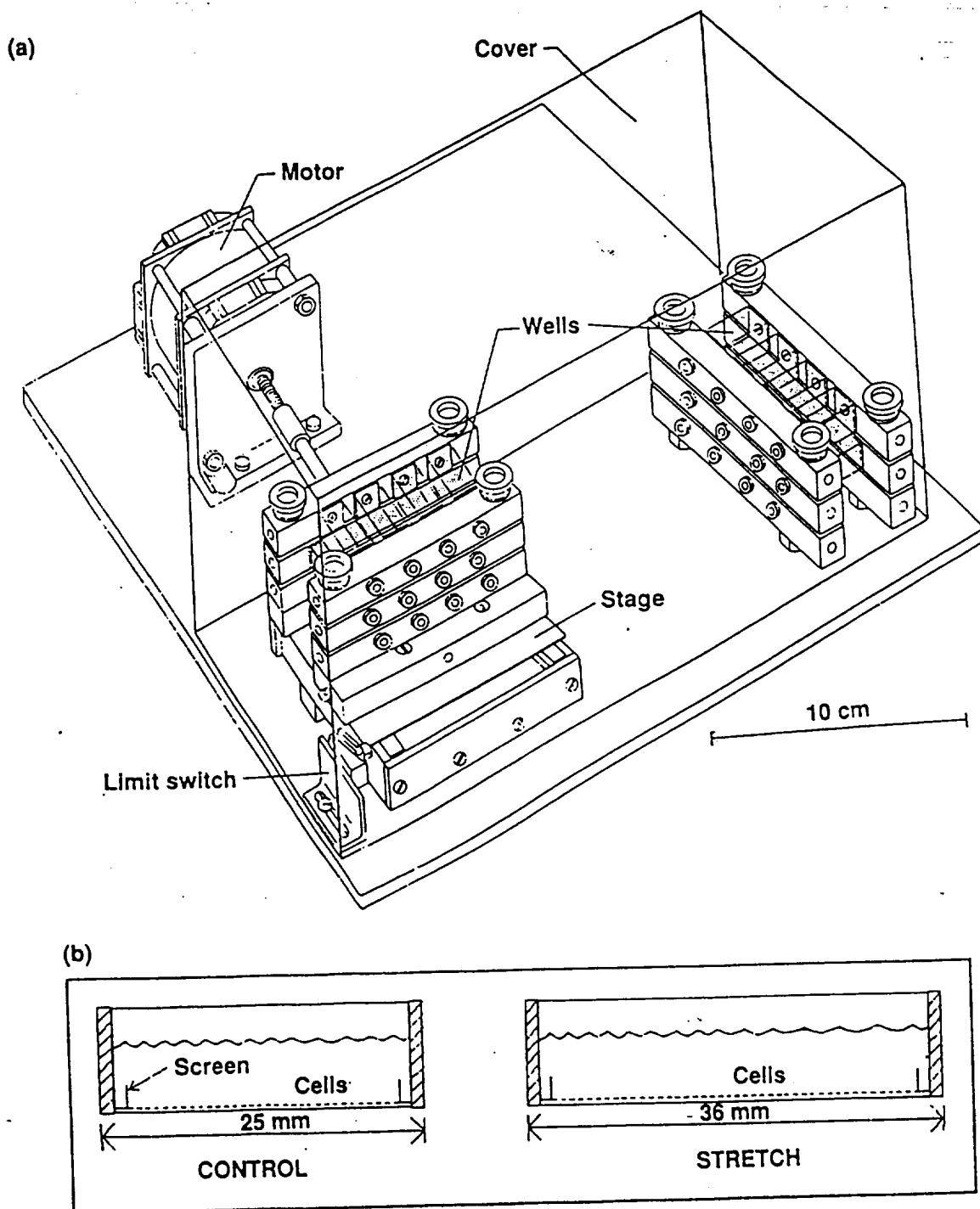
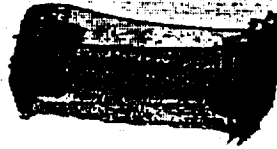
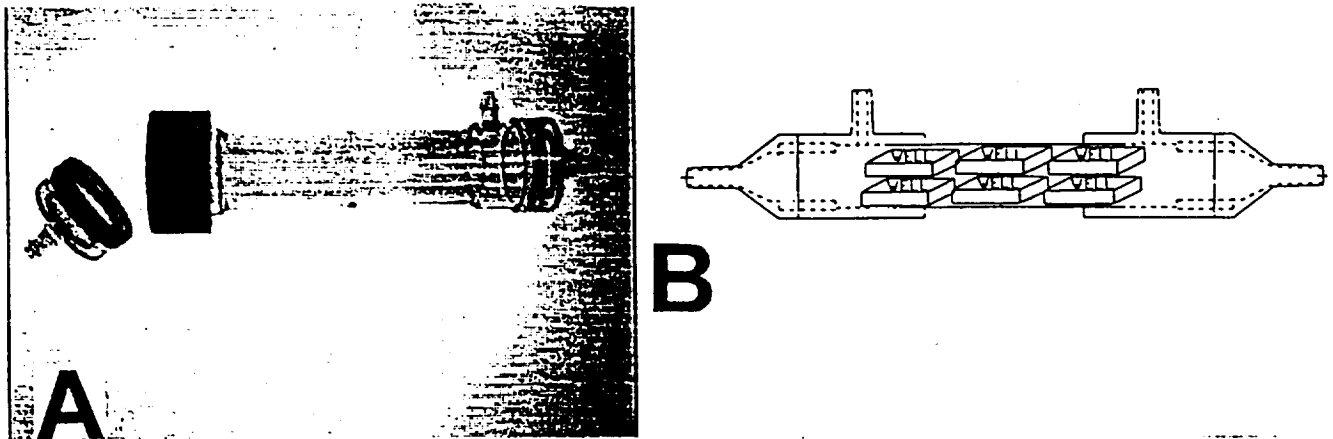


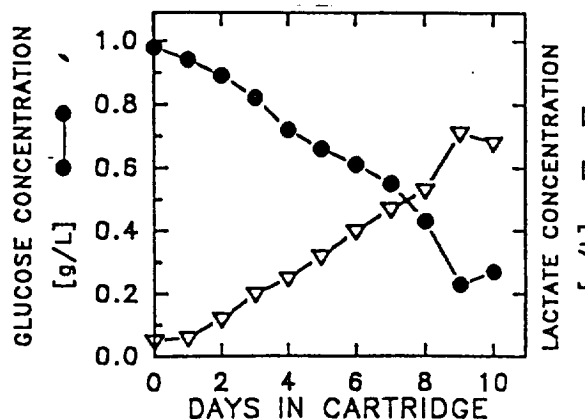
Fig. 3. Mechanical Cell Stimulator, Model II. (a) Perspective line drawing showing stepper motor, linear positioning translation stage (STAGE), control wells (right), and mechanically stretched wells (left). Wells are constructed of Silastic™ membrane. Limit switch automatically shuts off the motor if the stage travels beyond a set distance. A Plexiglass cover is placed over the wells to maintain sterility. Not shown in the diagram is the Apple IIe computer and connecting wires to the motor unit which control the activity pattern. (b) Enlarged side view of a culture well shows the stretching of the cells by horizontal movements of the elastic substratum and attached cells in one dimension. One end of each well is held in a fixed position while the other end is stretched. (See Vandenburg and Karlisch, 1989 for details).



**FIGURE 4.** Photograph of stainless steel wire mesh bracket inside of elastic culture well of MCS-2. Organoid (not seen in photo) is held at resting tension and are well protected by the elastic substratum and the bracket when inserted into modified cartridge of STL Module.



**FIGURE 5.** Modified cartridge of STL Module has a screw cap end (A) allowing insertion and removal of up to 6 skeletal muscle organoids (B). Wells are held tightly in place by friction.



**FIGURE 6.** Skeletal muscle organoids are metabolically active when placed in modified cartridges of the STL Module. In normal Basal medium Eagle's (1.0 g/L glucose), glucose in 100 ml of medium lasts approximately 8 days. Lactate buildup did not lead to a significant drop in medium pH. Glucose and lactate were measured daily in 500  $\mu$ l of medium using a Yellow Springs Clinical Glucose/Lactate Analyzer.

**E. Publications since last competitive renewal:****(1) Peer-reviewed papers:**

1. Vandemburgh, H.H., Karlisch, P., Shansky, J., and Feldstein, R. (1991) Insulin and insulin-like growth factor-1 induce pronounced hypertrophy of skeletal myofibers in tissue culture. Am. J. Physiol. **260**, C475-C484.
2. Vandemburgh, H.H., Swasdison, S., and Karlisch, P. (1991) Computer aided mechanogenesis of skeletal muscle organs from single cells *in vitro*. FASEB J. **5**, 2860-2867. (Cover Article)
3. Vandemburgh, H.H. (1992) Mechanical forces and their second messengers in stimulating cell growth *in vitro*. Am. J. Physiol. **262**, R350-R355.
4. Vandemburgh, H.H., Hatfaludy, S., P. Karlisch, and J. Shansky (1992) Mechanically induced alterations in cultured skeletal muscle growth. J. Biomech. **24**, 91-101.
5. Chromiak, J.A. and Vandemburgh, H.H. (1992) Glucocorticoid-induced skeletal muscle atrophy *in vitro* is attenuated by mechanical stimulation. Am. J. Physiol. **262**, C1471-C1477.
6. Vandemburgh, H.H., Shansky, J., Karlisch, P., and Solerssi, R. (1993) Mechanical stimulation of skeletal muscle generates lipid-related second messengers by phospholipase activation. J. Cell. Physiol. **155**, 63-71.
7. Chromiak, J.A. and Vandemburgh, H.H. (1993) Mechanical stimulation of skeletal muscle mitigates glucocorticoid-induced decreases in prostaglandin synthesis. J. Cell Physiol., In Press
8. Vandemburgh, H.H., Solerssi, R., Shansky, J., Adams, J., Henderson, S. (1993) Mechanical stimulation of organogenic cardiomyocyte growth *in vitro*. Circ. Res., In Revision.
9. Perrone, C.E., Fenwick-Smith, D., Vandemburgh, H.H. (1994) Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and its binding proteins from differentiated skeletal muscle cells. J. Biol. Chem., In Revision.
10. Vandemburgh, H.H., Shansky, J., Solerssi, R., Chromiak, J. (1994) Mechanical stimulation of skeletal muscle *in vitro* increases prostaglandin F<sub>2</sub> synthesis and cyclooxygenase activity by a pertussis toxin sensitive mechanism. Submitted.
11. Chromiak, J.C., and Vandemburgh, H.H. (1994) Anabolic/androgenic steroids alter protein turnover of tissue cultured avian skeletal muscle. In Preparation.

**(2) Other publications:**

1. Shansky, J., Karlisch, P., and Vandemburgh, H.H. (1993) Skeletal muscle mechanical cell stimulator. In Protocols in Cell and Tissue Culture (J.B. Griffiths, A. Doyle, and G. Newell, eds.), John Wiley and Sons Limited, Chichester, 11B:9.1-11B:9.7.

**(c) Abstracts:** 11 from 1991-1994

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